

# THE DETECTION AND SEPARATION OF PECTIC SUBSTANCES BY PAPER CHROMATOGRAPHY AND PAPER ELECTROPHORESIS

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The great interest in pectic substances makes the development of new techniques for detection and identification of these compounds highly desirable. However, the heterogeneity of these substances causes difficulties in their characterization.

Paper chromatography has been successfully applied for the separation of various degradation products of pectic substances. These degradation products are obtained by acid or enzymatic hydrolysis and more recently by heating with the cation exchange resin, Permutit Q, in a sealed tube.

The last method of hydrolysis yields a solution of galacturonic acid, oligogalacturonides, galactose, arabinose, and rhamnose as detected by chromatography.

A general chromatographic method for the detection and separation of pectic substances has not been developed till now. The aim of this paper is to present a method for chromatographic and electrophoretic separation on paper of different pectin preparations.

The method to be described was based on the following properties of pectic substances:

1. The relative solubility of pectinic acid (pectin) in water and its insolubility in ethanol suggested that either water (buffer) or a mixture of ethanol-water could be used as solvent for paper chromatography of pectin.

2. Pectin is thought to consist mainly of polygalacturonic acid esters. These should give similar reactions as carboxylic acid esters.

The use of hydroxylamine in alkaline solution to convert polygalacturonic acid esters into hydroxamates followed by ferric chloride as color reagent appeared to be promising for the detection of esterified polygalacturonic acid units (Whittaker and Wijesundera, 1951).

3. Pectic substances show a relatively high mobility in electrophoresis (Tiselius and Ingelman, 1942; Speiser, Copley and Nutting, 1947). This suggested that after the migration in an electric field the pectic substances could be detected by spraying the paper with hydroxylamine-ferric-chloride reagent.

## EXPERIMENTAL PROCEDURE

*Material and methods.*—The following substances were used: pectin N.F., pectin N.F. heated for 8 hours in 1 percent solution, 1 percent solution of pectin N.F. heated for 24 hours, pectin obtained from bean leaves by extraction with 0.5 percent of ammonium oxalate and radioactive pectin.

Radioactive pectin was prepared as follows:

Fourteen mature bean leaves, fresh weight 5.29 gms., were placed in a desiccator with petioles immersed in water.  $C^{14}O_2$  generated from 1 mg.  $Ba\ C^{14}O_3$  by lactic acid was passed into the desiccator, and the apparatus was allowed to remain in the light for 20 hours.

The leaves were then put into boiling 95 percent ethanol, boiled for 10 minutes, and dried in a vacuum oven for 5 hours at 60°C.

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The dried material was extracted with 0.5 percent ammonium oxalate three times for 6 hours.

Pectin was precipitated by adding 1 volume of acetone and 200 to 300 mg. percent of sodium chloride.

After 2 hours, the acetone-water layer was decanted and about 60 cc. of a colloidal suspension was centrifuged at 5000 r.p.m.

The residue was dissolved in 10 cc. of water, centrifuged and reprecipitated with 10 cc. of acetone.

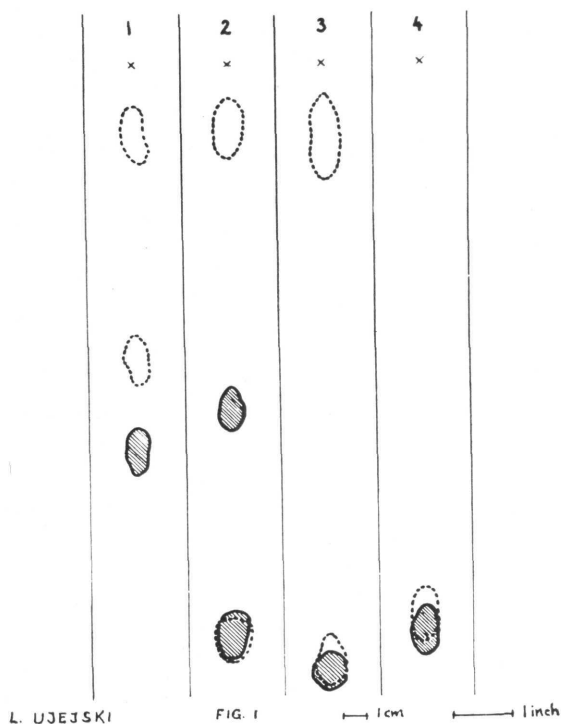


FIGURE 1. Paper chromatography of pectic substances

- (1) Pectin N.F.
- (2) Pectin N.F. heated for 8 hours
- (3) Pectin N.F. heated for 24 hours
- (4) Pectin obtained from bean leaves

Dotted circles represent the free acids



FIGURE 2.  
Autoradiogram  
of radioactive  
pectin

*Paper chromatography.*—No. 3 Whatman filter paper was washed with 0.3 N hydrochloric acid, water, 0.5 N sodium hydroxide, water. The chromatograms were developed with 33 percent ethanol in water v./v. by the descending technique, until the solvent front passed about 6 cm. beyond a pencil mark 3 inches from the upper edge (Boman, 1952). The paper was then quickly taken out, 25  $\mu$ l. spots of various samples were applied to the pencil mark and the migration continued for 3 hours (fig. 1 and 2).

*Reagents.*—

*Hydroxylamine.* Solution **A**: 20 gm. of hydroxylamine hydrochloride were dissolved in 50 ml. of water and diluted to 200 ml. with ethanol; Solution **B**:

50 gm. of potassium hydroxide in a minimum of water were diluted to 500 ml. with ethanol. After mixing the solutions A and B, potassium chloride was removed.

*Ferric chloride.* 10 gm. of  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  were dissolved in 20 ml. of 10 N HCl and the solution shaken with 300 ml. of ether until homogeneous.

The strips were sprayed with ethanolic solution of hydroxylamine, dried at room temperature for 15 minutes, then dipped into an ethereal acid solution of ferric chloride and immediately washed under tap water until the background became white.

Well developed purple spots were formed showing the position of esters.

A spray, consisting of a solution of 50 mg. bromophenol blue in 100 mg. of 95 percent ethanol adjusted to pH 6.5 with sodium hydroxide, revealed the positions occupied by free acids.

*Paper electrophoresis.*—Spinco paper electrophoresis cell, Durrum type, and strips of Whatman 3 mm. filter paper 30 mm. wide were used. The strips were saturated with acetate buffers (0.2M solution) of different pH values: 3.6, 4, 4.6, 4.9, 5.5.

0.01 ml. of 1 percent solutions were applied to strips.

Satisfactory migration was observed (fig. 3) when a constant voltage of 200 V, D.C., was maintained for three and one-half hours.

The bands were then detected by means of hydroxylamine-ferric chloride reagent (fig. 3).

No migration was observed at pH 5.5.

#### RESULTS AND DISCUSSION

Thirty-three percent ethanol-water solution used as solvent for development of chromatograms of pectic substances combined with the technique of Boman (1952) appeared to be the most suitable.

Besides this solvent, numerous other solvent systems were tried. Glycerol-water, glycerol-ethanol, dioxane-water, furfuryl alcohol-water, pyridine-water produced tailing spots.

Better chromatograms were obtained with 12 percent ethanol-water with addition of 200 mg. of NaCl.

Hydroxylamine-ferric chloride reagent gives well defined spots with amounts as low as 30 micrograms of pectin.

The chromatograms sprayed with the hydroxylamine-ferric chloride reagent displayed the following spots (fig. 1).

Strip No. 1 is a chromatogram of unheated 1 percent solution of pectin N.F. It shows one slow moving spot.

Strip No. 2 is a chromatogram of the same pectin solution but heated 8 hours on a water bath. It displays two spots. The slower moving spot has the same rate of migration as the spot on strip No. 1.

Strip No. 3 is a chromatogram of 1 percent solution of pectin N.F. heated for 24 hours on a water bath. This strip shows a single fast moving spot whose position corresponds to that of the fast moving spot of strip No. 2.

Strip No. 4 represents the chromatogram of pectin obtained from bean leaves. Again there is only one spot corresponding in position to that of the fast moving spot on strips No. 2 and 3.

Figure 2 is an autoradiogram of radioactive pectin. The rate of migration of the fast moving spot is similar to that of strip No. 3.

The differences in movement on paper of heated and unheated samples are probably due to physio-chemical changes in pectin macro-molecules.

It has been generally observed that heated pectin solutions exhibit a rapid decrease in viscosity. According to the theory of Kertesz (1951), the structure of

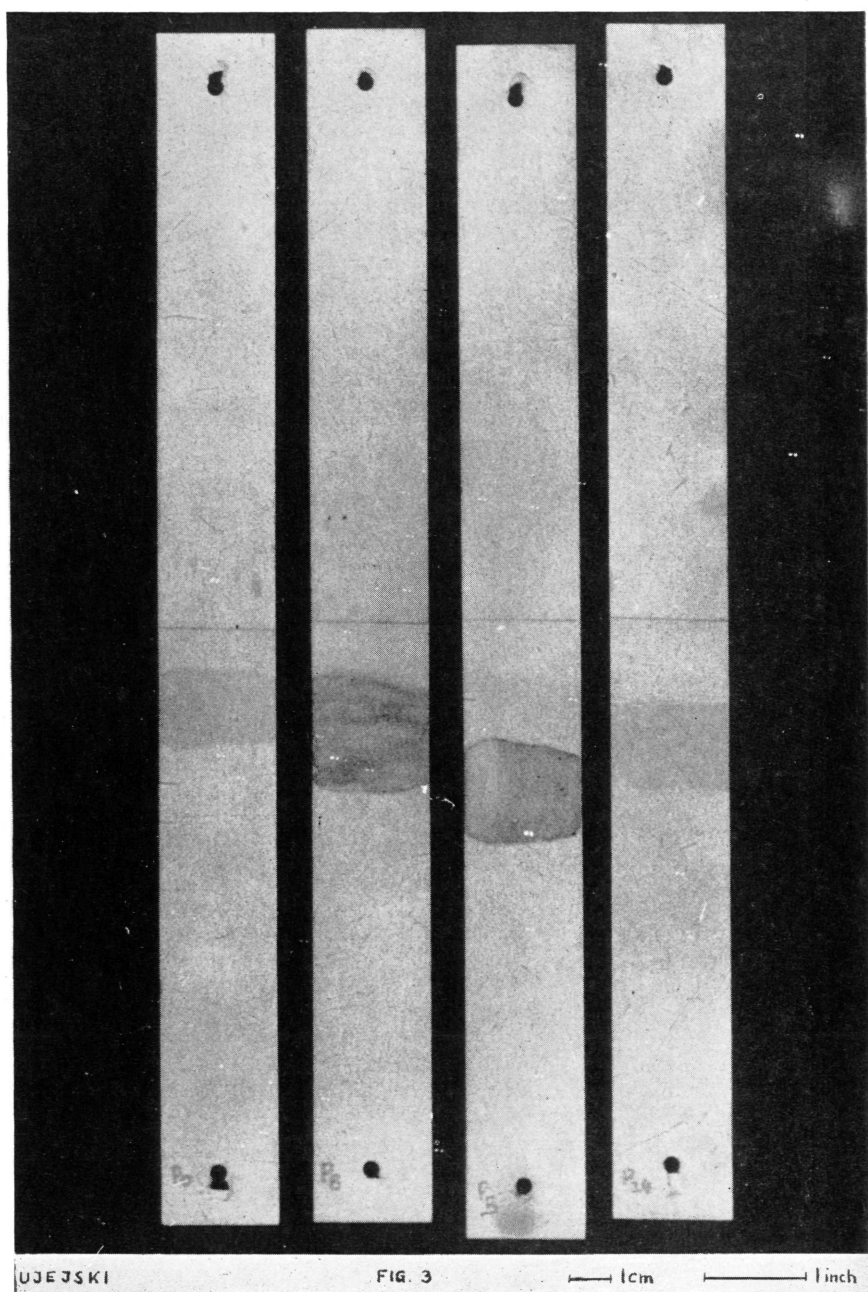


FIGURE 3. Paper electrophoresis of pectic substances

- (A) Pectin N.F.
- (B) Pectin N.F. heated for 8 hours
- (C) Pectin obtained from bean leaves
- (D) Pectin N.F. heated for 24 hours

pectin solutions might be expressed by the formula  $[(Gm)]^n$ , where Gm represents a polymer of  $m$  galacturonic acid units and forms aggregates containing  $n$  of these units held together by secondary valence forces. These secondary aggregates are thought to be mainly responsible for the high viscosity of pectin solutions, whereas losses of viscosity occurring during heating are due to the destruction of these aggregates held by secondary forces.

In agreement with this theory, the slow moving spots on the chromatograms of unheated and heated solutions (8 hours) might correspond to the secondary aggregates, and the fast moving ones to the molecules after disruption of the secondary valence forces.

Dotted circles on figure 1 represent the detection of free acids in different pectin preparations by means of bromophenol blue reagent.

All strips except No. 4 (pectin from bean leaves) and radioactive pectin show two yellow spots on blue background. The position of the second faster moving spot is the same as that of the ester units; this would indicate a partial deesterification.

Figure 3 shows electrophorograms of the pectin preparations.

The method herein described is the first successful adaptation of conventional paper chromatography and paper electrophoresis to the detection and separation of pectic substances. It is believed that it represents useful techniques for the characterizing of these scientifically and commercially important substances.

#### SUMMARY

A new procedure is presented for the detection and separation of pectic substances which have been separated by paper chromatographic and electrophoretic methods.

Hydroxylamine in alkaline solution, converting polygalacturonic acid esters into hydroxamates followed by ferric chloride as color reagent, was used for the detection of pectic substances on paper.

Bromophenol blue (pH=6.5) was applied for location of free carboxylic acids.

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#### LITERATURE CITED

- Boman, H. G. 1952. Double fronting in paper chromatography of proteins. *Nature* 170: 703.  
Kertesz, Z. I. 1951. *The Pectic Substances*. p. 53. Inter-science Publisher Inc., New York.  
Speiser, R., M. J. Copley, and G. C. Nutting. 1947. Effect of molecular association and charge distribution on the gelation of pectin. *Jour. Phys. and Colloid. Chem.* 51: 117.  
Tiselius, A. and B. Ingelman. 1942. *Förh. Svenska Socker*, II, 16 pp.  
Whittaker, V. P. and S. Wijesundera. 1951. Separation of esters of choline by filter-paper-chromatography. *Biochem. Soc. Proc.* p. xvi (bound with *Biochem. Jour.* vol. 49).